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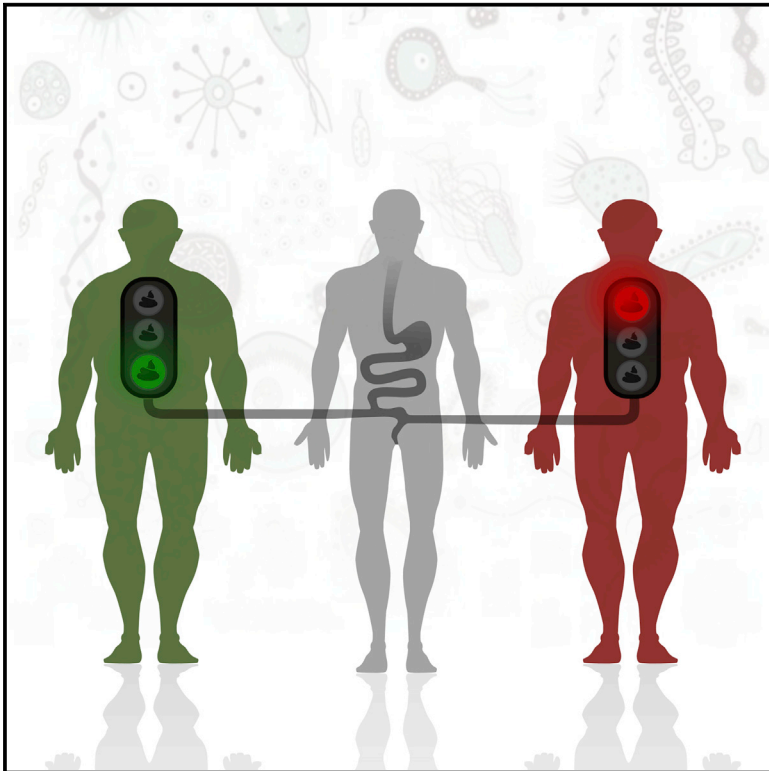
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Cell Metabolism

Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition

Graphical Abstract



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In Brief

Kootte et al. show that fecal microbiota transplantation from lean donors to obese patients with metabolic syndrome improves insulin sensitivity, a transient effect associated with changes in microbiota composition and fasting plasma metabolites. Baseline fecal microbiota composition in recipients predicts the response to lean donor fecal microbiota transplantation.

Highlights

- Lean donor FMT in obese metabolic syndrome patients improves insulin sensitivity
- Beneficial effects of lean donor FMT are transient
- Improvement in insulin sensitivity is linked to changes in plasma metabolites
- Response to lean donor FMT is driven by baseline fecal microbiota composition



Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition

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SUMMARY

The intestinal microbiota has been implicated in insulin resistance, although evidence regarding causality in humans is scarce. We therefore studied the effect of lean donor (allogenic) versus own (autologous) fecal microbiota transplantation (FMT) to male recipients with the metabolic syndrome. Whereas we did not observe metabolic changes at 18 weeks after FMT, insulin sensitivity at 6 weeks after allogenic FMT was significantly improved, accompanied by altered microbiota composition. We also observed changes in plasma metabolites such as γ -aminobutyric acid and show that metabolic response upon allogenic FMT (defined as improved insulin sensitivity 6 weeks after FMT) is dependent on decreased fecal microbial diversity at baseline. In conclusion, the beneficial effects of lean donor FMT on glucose metabolism are associated with changes in intestinal microbiota and

plasma metabolites and can be predicted based on baseline fecal microbiota composition.

INTRODUCTION

The worldwide burden of obesity and related illnesses such as diabetes mellitus warrants new therapeutical modalities, as current therapies such as lifestyle changes and antidiabetic agents are insufficiently capable of reducing morbidity and mortality rates (Cefalu et al., 2015). During the last decade, changing the composition of intestinal microbiota has gained attention as a novel therapeutic modality to improve insulin sensitivity (Khan et al., 2014). Several lines of evidence have indicated that the bacterial composition differs between lean and obese animals (Ley et al., 2005), but also that gut microbial composition may reflect an aberrant metabolic function including altered digestion of dietary products (Turnbaugh et al., 2006). Finally, these animal studies have suggested a causal relation since the adverse phenotype is transferable via fecal transplantation (Bäckhed et al., 2004; Turnbaugh et al., 2006). Although many

Table 1. Baseline Characteristics

	Autologous FMT (n = 12)	Allogenic FMT (n = 26)	p Value
Male gender (%)	100	100	1.000
Age (years)	54 [49–58]	54 [49–60]	0.914
BMI (kg/m ²)	35.8 [33.1–40.4]	33.8 [32.5–35.7]	0.093
Waist circumference (cm)	121 [115–130]	120 [111–127]	0.297
Blood pressure: systolic (mmHg)	148 [134–162]	141 [132–154]	0.243
Blood pressure: diastolic (mmHg)	94 [83–105]	90 [78–97]	0.218
Fasting glucose (mmol/L)	5.9 [5.5–6.4]	5.5 [5.3–6.1]	0.187
HbA1c (mmol/mol)	43 [36–46]	40 [36–42]	0.218
Insulin (pmol/L)	107 [80–159]	121 [93–143]	0.722
HOMA-IR	4.8 [3.5–6.9]	4.8 [3.7–6.0]	0.963
Cholesterol: total (mmol/L)	5.5 [4.8–6.6]	5.5 [4.8–6.6]	0.865
Cholesterol: LDL (mmol/L)	3.7 [3.0–4.8]	3.9 [3.2–4.5]	0.914
Cholesterol: HDL (mmol/L)	1.0 [0.9–1.1]	1.1 [0.9–1.4]	0.297
Cholesterol: triglycerides (mmol/L)	1.3 [1.1–1.8]	1.2 [0.9–1.7]	0.485

Baseline characteristics for patients randomized for autologous and allogenic (both one and two) treatment(s), expressed as medians and interquartile ranges in brackets. $p < 0.05$ was considered significant. See also Table S1. BMI, body mass index; FMT, fecal microbiota transplantation; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment – insulin resistance; LDL, low-density lipoprotein.

observational studies have suggested correlations between altered microbiota composition and metabolism in humans, causality has been difficult to prove (Smits et al., 2013). In this respect, we have previously shown in a small pilot study that transplantation of lean donor fecal microbiota into obese males with metabolic syndrome resulted in improved glucose metabolism, which was associated with alterations in intestinal microbiota composition in both fecal and duodenal samples (Vrieze et al., 2012). These findings prompted us to study the short- and long-term effect of lean donor fecal microbiota transplantation (FMT) on gut microbiota composition in a larger group of male metabolic syndrome subjects and dissect the underlying pathophysiology of insulin resistance by relating intestinal microbiota changes to various markers of metabolism. Moreover, we tried to unravel whether specific characteristics of fecal microbiota composition at baseline can explain why some metabolic syndrome subjects improve in insulin sensitivity upon lean donor FMT treatment (so-called metabolic responders), whereas others do not (non-responders).

RESULTS AND DISCUSSION

In line with several recent observational studies that have supported a role for the intestinal microbiota in metabolic processes

(Thaiss et al., 2016; Zhernakova et al., 2016), and in accordance with our previous pilot study (Vrieze et al., 2012), we report that altering intestinal microbiota composition by infusion of healthy lean donor feces has a (short-term) beneficial impact on peripheral insulin sensitivity in metabolic syndrome patients. In contrast to animal studies, this effect on insulin-mediated glucose uptake was not associated with changes in (post-prandial) incretins or bile acids (Sayin et al., 2013; Wichmann et al., 2013). Other observations point toward a regulatory role of plasma metabolites that are derived from diet and converted by intestinal microbiota in insulin-resistant obese males (Guasch-Ferré et al., 2016). Although the overall metabolic effects of lean donor FMT are modest and show a wide variety between patients, our data do suggest that changes in plasma metabolites (predominantly amino acids), as a consequence of the altered intestinal microbiota composition, might be one explanation for the beneficial effects of lean donor FMT on peripheral insulin sensitivity.

Inclusion

We included 44 male metabolic syndrome subjects, of whom 6 were excluded (three due to pathology of the proximal gastrointestinal tract for which immediate treatment was warranted, found during the first gastroduodenoscopy [two ulcer ventriculi and one Barrett esophagus with dysplasia]; one subject withdrew from the study after the first treatment week; and two subjects were excluded due to technical difficulties with several clinical measurements). Therefore, 38 subjects were enrolled in this study and were randomly distributed over all treatment groups (Tables 1 and S1). We found no differences in baseline characteristics between recipients of autologous and allogenic FMT. We included 11 healthy lean donors, who provided their fresh feces for the metabolic syndrome subjects randomized for allogenic FMT ($n = 26$). Seven lean donors provided feces for multiple metabolic syndrome subjects with a range of two to five recipients per single lean donor, while four lean donors provided feces for one allogenic FMT. Throughout the follow-up period, there were no serious adverse events or adverse changes in plasma biochemistry.

Long-Term Effects of Lean Donor FMT

We found that duodenal and fecal microbiota composition at 18 weeks after allogenic FMT was similar to baseline (Figures 1A and 1B). In line, neither single nor repeated allogenic FMT had significant effects on hepatic (expressed as insulin-mediated suppression of endogenous glucose production; EGP) or peripheral insulin sensitivity (expressed as rate of glucose disappearance; Rd) at 18 weeks (Figure S1). Weight (plasma and fecal), short-chain fatty acids (SCFAs), and fasting plasma metabolites (Figure S2) were not affected. The lack of long-term clinical effects (both on insulin sensitivity and plasma metabolites) at 18 weeks after allogenic FMT in our study is in line with previous findings on transient donor bacterial strain engraftment and the presence of a personal fecal core microbiome (Li et al., 2016). In this regard, it is likely that the host immune system develops resilience, which, in combination with the adherence of one's own lifestyle, including diet (Zeevi et al., 2015), could explain the return of intestinal microbiota composition to the baseline situation and the magnitude of metabolic response both at short- and long-term (Marques et al., 2016a).

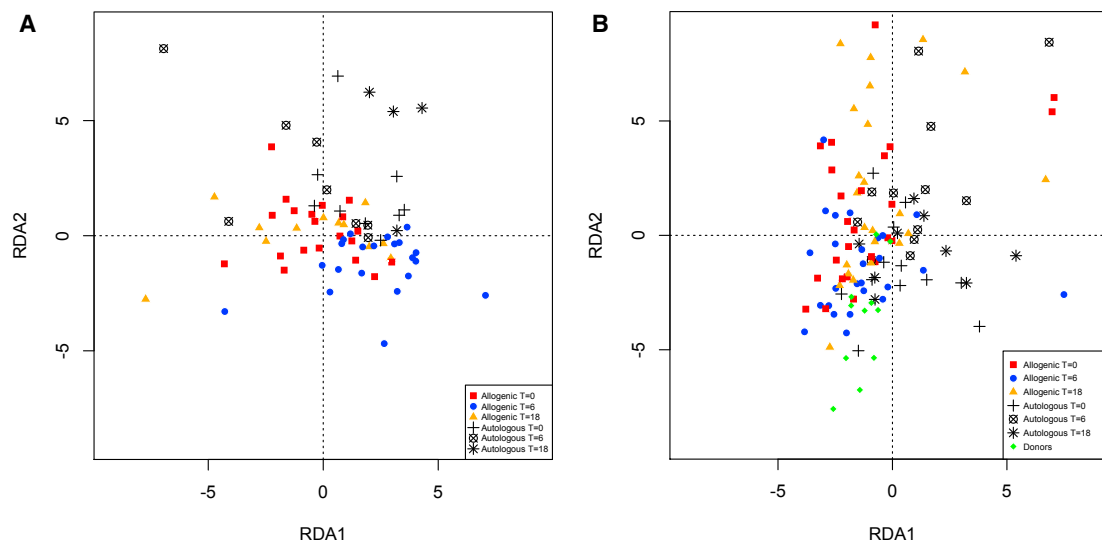


Figure 1. Changes in Duodenal and Fecal Microbiota Composition after Autologous and Allogenic FMT

(A) Biplot of redundancy analysis (RDA axis 1 versus axis 2) of duodenal microbiota data, constrained by autologous and allogenic treatment variables in time (0, 6, and 18 weeks).

(B) Biplot of redundancy analysis (RDA axis 1 versus axis 2) of fecal microbiota data, constrained by autologous and allogenic treatment variables in time (0, 6, and 18 weeks). Also, baseline fecal microbiota composition in lean FMT donors ($n = 11$) is depicted.

FMT, fecal microbiota transplantation.

Short-Term Effects of Lean Donor FMT: Insulin Sensitivity and Post-prandial Metabolism

As we did not observe long-term changes in these major outcome parameters, we focused on changes between weeks 0 and 6 and found that allogenic FMT did result in altered duodenal and fecal microbiota composition at 6 weeks (Figures 1A and 1B). This was associated with improved peripheral insulin sensitivity at week 6 (from $25.8 [19.3\text{--}34.7]$ to $28.8 [21.4\text{--}36.9]$ $\mu\text{mol kg}^{-1} \text{min}^{-1}$, $p < 0.05$), whereas autologous FMT had no effect (from $22.5 [16.9\text{--}30.2]$ to $20.8 [17.6\text{--}29.5]$ $\mu\text{mol kg}^{-1} \text{min}^{-1}$, n.s.) (Figure 2; Table S2). The change in peripheral insulin sensitivity in the allogenic FMT group was accompanied by a small but significant decrease in glycated hemoglobin (HbA1c) at 6 weeks ($39.5 [36.0\text{--}41.0]$ to $38.0 [34.0\text{--}41.0]$ mmol/mol, $p < 0.01$). Other metabolic parameters (e.g., weight, caloric intake, and resting energy expenditure [REE]) were not affected (Tables S2 and S3). Although the results were more variable than in our previous smaller study, we were able to reproduce this previous finding of short-term beneficial changes on peripheral insulin sensitivity upon allogenic FMT (Vrieze et al., 2012). These data concur with our previous data that insulin resistance in obese subjects is characterized by a more pronounced decrease in peripheral insulin sensitivity, whereas hepatic insulin sensitivity is affected to a lesser and more variable extent (ter Horst et al., 2015). Moreover, upon administration of insulin-sensitizing medication, peripheral insulin sensitivity improved significantly more than hepatic insulin sensitivity (Serlie et al., 2007). In line, we could discern that approximately half of all subjects treated by allogenic FMT showed a clinically relevant improvement at 6 weeks. This modest therapeutic effect and large variability on whole-body glucose metabolism upon targeted intestinal microbiota intervention is less pronounced than in mice (Bäckhed et al., 2004),

but aligns with our previous publications on the metabolic effects of oral antibiotics to specifically alter intestinal microbiota composition (Vrieze et al., 2014; Reijnders et al., 2016). With regard to post-prandial metabolism, the allogenic FMT group showed an increased post-prandial rise in plasma triglycerides (incremental area under the curve [IAUC] = $91 [52\text{--}129]$ to $115 [77\text{--}146]$ mM/min, $p < 0.05$) between weeks 0 and 6. Other markers such as glucose and enteroendocrine hormones (e.g., GLP-1, GIP, and PYY) were not influenced by allogenic FMT (data not shown). As was described in previous reports, we found (minor) changes in fecal bile acids (predominantly cholate) (Weingarden et al., 2014) upon allogenic FMT. Although allogenic FMT did not affect fasting and post-prandial total plasma bile acid concentrations, allogenic FMT significantly increased fecal cholate excretion (both for fecal concentration and fecal excretion, $p < 0.05$) (Figure S3).

Short-Term Effects of Lean Donor FMT: SCFAs, Intestinal Microbiota, and Plasma Metabolites

Fasting plasma SCFA levels did not change upon allogenic FMT, and in contrast to our previous study (Vrieze et al., 2012), we did not observe significant changes in fecal butyrate levels (butyrate from $13 [6\text{--}27]$ to $20 [13\text{--}27]$ $\mu\text{mol/g feces}$, $p = 0.096$) (Table S3). Fecal acetate levels, however, were significantly increased from $62 [43\text{--}89]$ to $85 [61\text{--}105]$ $\mu\text{mol/g feces}$ ($p < 0.05$) after allogenic FMT, whereas fecal propionate was borderline significantly altered (from $23 [16\text{--}35]$ to $28 [22\text{--}60]$ $\mu\text{mol/g feces}$, $p = 0.062$). Subsequent microbiota analyses showed that allogenic treatment resulted in significantly altered duodenal bacterial species including *Bifidobacterium pseudolongum*, previously mentioned as a potential probiotic that produces acetate from dietary carbohydrates (Figure 3A) (Pokusaeva et al., 2011). In line with our findings of increased fecal acetate upon allogenic

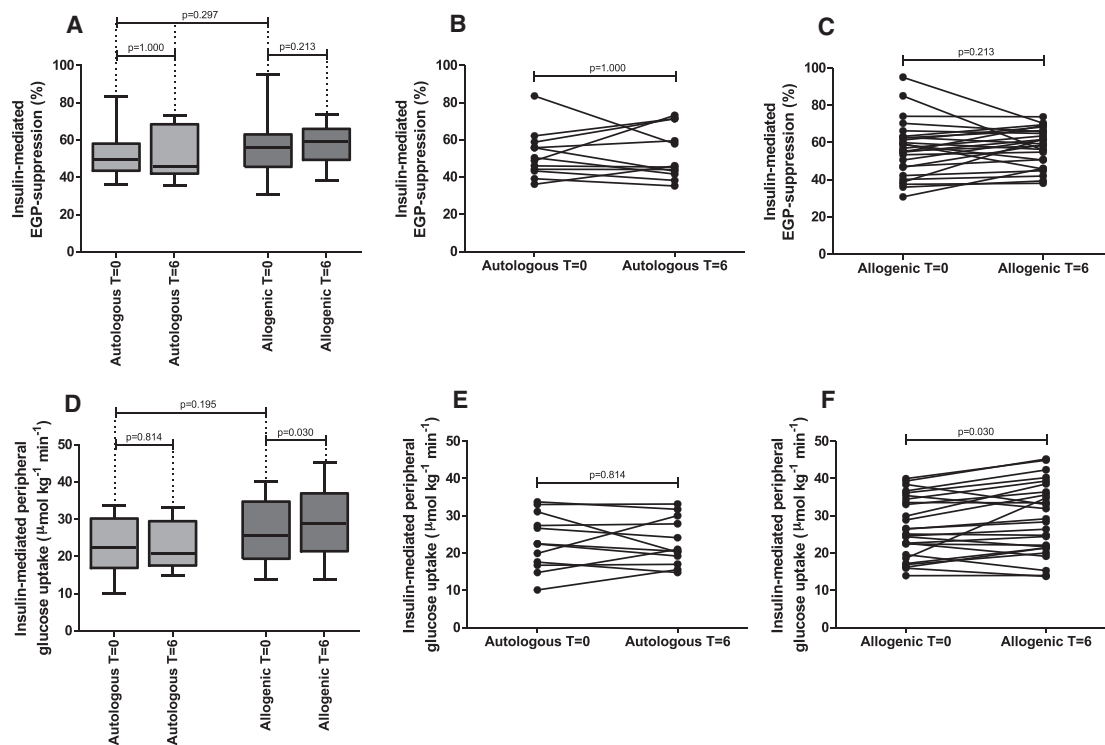


Figure 2. Changes in Hepatic and Peripheral Insulin Sensitivity between Weeks 0 and 6

This figure shows changes in insulin sensitivity between weeks 0 and 6 for both the autologous and allogenic treatment group. Insulin sensitivity is described as percentage suppression of EGP as a marker of hepatic insulin sensitivity (A–C), as well as a marker of peripheral insulin sensitivity (D–F). Grouped data are expressed as box-and-whisker plots, whereas the spaghetti plots show individual data. $p < 0.05$ was considered significant. See also Table S2. EGP, endogenous glucose production; FMT, fecal microbiota transplantation.

FMT, fecal acetate levels are inversely related to insulin resistance in humans (Yamaguchi et al., 2016). Several fecal bacterial species that were different between autologous and allogenic FMT have been linked to human metabolism (Figure 3B). These include the lactate-producing *Lactobacillus salivarius* (Mesaoudi et al., 2013) and butyrate-producing *Butyrivibrio* (de la Cuesta-Zuluaga et al., 2017), *Clostridium symbiosum* (O’Keefe et al., 2015), and *Eubacterium* species (Vrieze et al., 2012; Udayappan et al., 2016). There were no differences in fecal microbial diversity (Shannon index) between baseline and 6 weeks (allogenic FMT group from 5.9 [5.8–6.0] to 6.0 [5.8–6.1], $p = 0.493$; autologous FMT group from 5.9 [5.8–6.1] to 6.0 [5.8–6.1], $p = 0.239$). Since both our smaller pilot study (Vrieze et al., 2012) and the current study showed a significant clinical effect of lean donor FMT on peripheral insulin sensitivity, we conclude that the differential presence of either acetate or butyrate producers in donor feces as well as sample size may both account for the observed differences. Although the driving factors of donor bacterial strain engraftment are currently unknown, we speculate that the level of metabolic response might be due to donor-host interactions. Whether and to what extent adding a standard dietary intervention, together with better matching of donors with hosts, could work synergistically on beneficial gut microbiota changes and metabolic response require further study.

With respect to other fasting plasma metabolites, intestinal microbiota have recently been linked to altered (branched chain) amino acid production in relation to dietary composition (Peder-

sen et al., 2016; Sonnenburg and Bäckhed, 2016). We observed significant changes in 30 metabolites associated with either allogenic FMT (positive weight, $n = 17$) or autologous FMT (negative weight, $n = 13$) (Figure 3C). Whereas allogenic FMT was mostly associated with changes in amino acid concentrations, autologous FMT was mostly associated with changes in oxidative stress and lipid-related metabolites. Among others, allogenic FMT resulted in changes in amino acids such as γ -aminobutyric acid (GABA), tryptophan, and kynurenine (involved in serotonin metabolism), as well as phenylalanine. In particular, we found that GABA levels showed the strongest relation with allogenic FMT at 6 weeks. Indeed, GABA has been associated with control of metabolism in murine models (Meng et al., 2016). In this regard, the observed change in *Lactobacillus brevis* might be of specific interest, since this bacterial species has been linked to GABA production (Yunes et al., 2016) and its supplementation in insulin-resistant rats improved glucose homeostasis (Marques et al., 2016b). Also, GABA supplementation can positively affect insulin sensitivity in rodents (Tian et al., 2011), which may relate to the beneficial effects on Rd. Based on the small sample size, however, we refrained from associating changes in plasma metabolites with Rd in our study.

Short-Term Effects of Lean Donor FMT: Responders versus Non-responders

Although at 6 weeks an overall significant improvement in peripheral insulin sensitivity was observed in the whole allogenic

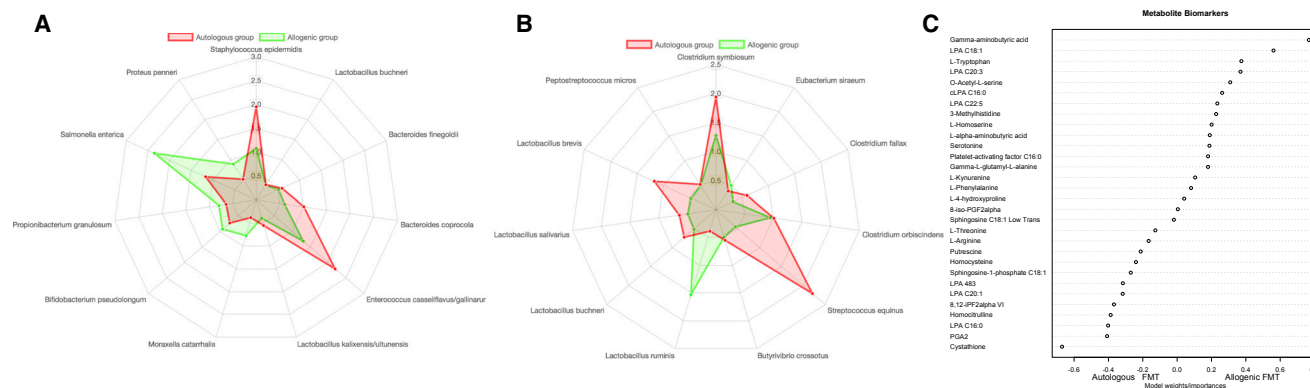


Figure 3. Changes in Microbiota Species and Plasma Metabolites after Autologous and Allogenic FMT

(A and B) The spider plot depicts a panel of bacterial species that significantly differentiate between autologous and allogenic treatment group, based on the changes in duodenal (A) and fecal (B) microbial composition 6 weeks after FMT. The axis of the spider plot reflects the amount of change (L2 norm) of the bacterial species upon autologous (red) or allogenic (green) FMT.

(C) Importance plot showing significant associations between changes in fasting plasma metabolites between weeks 0 and 6 and either autologous or allogenic FMT. Plasma metabolites are depicted on the y axis. Weights per metabolite are expressed on the x axis: a positive weight (n = 17) represents an association with allogenic lean donor feces treatment, whereas a negative weight (n = 13) represents an association with autologous feces treatment. The higher the weight, the stronger the association.

FMT, fecal microbiota transplantation; IPF, isoprostan F; LPA, lysophosphatidic acid; PGA, prostaglandin A2; PGF, prostaglandin F.

FMT group, a large variation in treatment efficacy was seen (Figures 2E and 2F), in line with earlier results reporting an inter-session coefficient of variance (CV) of 10% in Rd (Vrieze et al., 2012; Reijnders et al., 2016). We therefore split the allogenic FMT-treated subjects into a group with an Rd increase $\geq 10\%$ (responders, n = 13) and a group with an Rd increase $< 10\%$ (non-responders, n = 13) between weeks 0 and 6. Within the responder group, both peripheral (median Rd from 29.9 [17.9–36.4] to 36.4 [25.3–41.3] $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p < 0.01$) and hepatic insulin sensitivity (median insulin-mediated EGP suppression from 54.9% [44.7–64.4] to 63.5% [48.5–68.8], $p < 0.05$) increased significantly, whereas in the non-responder group no effect was seen. Weight did not change in either of the two groups. Within the responders, we observed a significant change in fecal abundance of *Akkermansia muciniphila* (Figures 4A and 4B), which has been linked to beneficial metabolic effects in targeted intervention studies in both rodents and humans (Shin et al., 2014; Dao et al., 2016). Moreover, our findings of alterations in SCFA-producing bacteria from the genus *Eubacterium* in relation to changes in insulin sensitivity are in line with findings in large cohorts of insulin-resistant subjects (Forslund et al., 2015; Dao et al., 2016). In contrast, the genus *Roseburia* was previously shown to be negatively correlated with an insulin-resistant state (Forslund et al., 2015), which is in contrast to our observation of overall decreased presence of this bacterial strain despite improved glucose regulation upon allogenic FMT. No significant changes were observed in Shannon's diversity index after allogenic FMT (responders from 5.8 [5.8–5.9] to 6.0 [5.8–6.1] versus non-responders from 6.0 [6.0–6.2] to 6.0 [5.9–6.1], n.s.) (Figure 5A).

Finally, we determined if fecal microbiota composition at baseline would be able to predict the responder or non-responder status upon lean donor allogenic FMT. With a good prediction (receiver operating characteristic [ROC] AUC 0.88; Figure 5B), we found that metabolic responders were character-

ized by lower initial fecal microbiota diversity (Figure 5A). This was combined with higher abundance of *Subdoligranulum variabile* and *Dorea longicatena* in comparison to non-responders, whereas abundance of *Eubacterium ventriosum* and *Ruminococcus torques* was lower in baseline fecal samples of responders (Figure 5C). Our findings thus confirm that lower fecal microbial diversity at baseline is predictive of metabolic improvement upon treatment (Cotillard et al., 2013; Le Chatelier et al., 2013). We also identified that the majority of the predictive fecal microbiota comprised abundance of four different species. In line with previous reports on metabolic response upon dietary intervention, our responders were characterized by increased pre-treatment abundance of *Subdoligranulum variabile* (Chum-pitazi et al., 2014). In contrast, the increased abundance of the species *Ruminococcus torques* in the baseline fecal samples of non-responders has been previously linked to adverse intestinal health (Png et al., 2010) and aberrant production of (fatty acid chain-containing) metabolites (Druart et al., 2014). Based on these findings, we conclude that for future interventions, determining baseline fecal microbiota composition might aid in predicting efficacy of treatment.

Limitations

Our study has some limitations. First, we chose to closely monitor caloric intake while keeping the regular diet of each study subject, as the introduction of a standardized diet influences gut microbiota composition (Zeevi et al., 2015). This could have influenced the impact of the FMT success. Second, our study was performed in male obese Caucasian subjects, possibly precluding generalization of our findings to other patient groups. Third, we used multiple fecal donors, which might explain the transient and variable effects seen in Rd upon allogenic FMT at 6 and 18 weeks. However, using one fecal donor was logistically not possible as we used fresh feces and no existing data were available, when our study was designed,

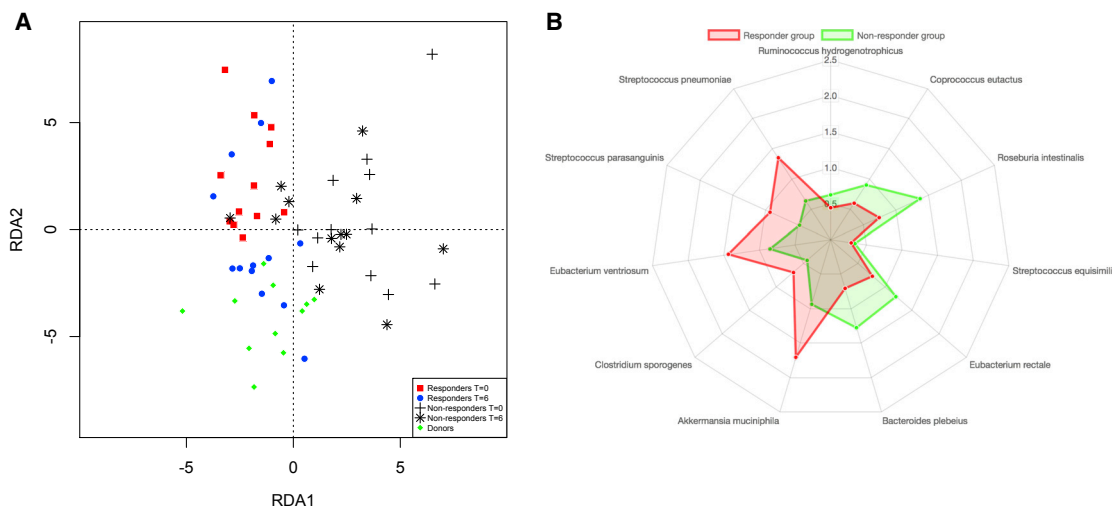


Figure 4. Changes in Fecal Microbiota Composition between Responders and Non-responders

(A) Biplot of redundancy analysis (RDA axis 1 versus axis 2) of fecal microbiota data, constrained by response and non-response variables in time (0 and 6 weeks). Response is defined by the Rd change 6 weeks after allogenic FMT, either $\geq 10\%$ increase (responders) or $<10\%$ increase (non-responders).

(B) Spider plot of bacterial species that significantly differentiate between responders and non-responders, based on the changes in fecal microbial composition 6 weeks after allogenic FMT. The axis of the spider plot reflects the amount of change (L2 norm) of the bacterial species in the responder (red) and non-responder (green) groups. Response is defined by the Rd change 6 weeks after allogenic FMT, either $\geq 10\%$ increase (responders) or $<10\%$ increase (non-responders). FMT, fecal microbiota transplantation; Rd, rate of (glucose) disappearance.

regarding matching criteria that would justify fecal donor stratification. Moreover, due to ethical constraints we were only allowed to determine Rd on three occasions, whereas in hindsight having a 12-week time point would have been a valuable addition. Nevertheless, our intervention study provides further evidence on the involvement of gut microbiota in human insulin resistance as single allogenic FMT (without previous antibiotic intestinal decontamination) resulted in a significant, short-term, beneficial therapeutic effect. Moreover, we were able to show

that the overall lack of long-term clinical effect (e.g., improvements in insulin sensitivity) was associated with return to baseline of both intestinal microbiota and plasma metabolites.

Conclusion

In conclusion, our data underscore the potential role of intestinally produced metabolites as (signaling) molecules and drivers of insulin sensitivity and underscore previous suggestions that pre-treatment fecal microbiota signatures might

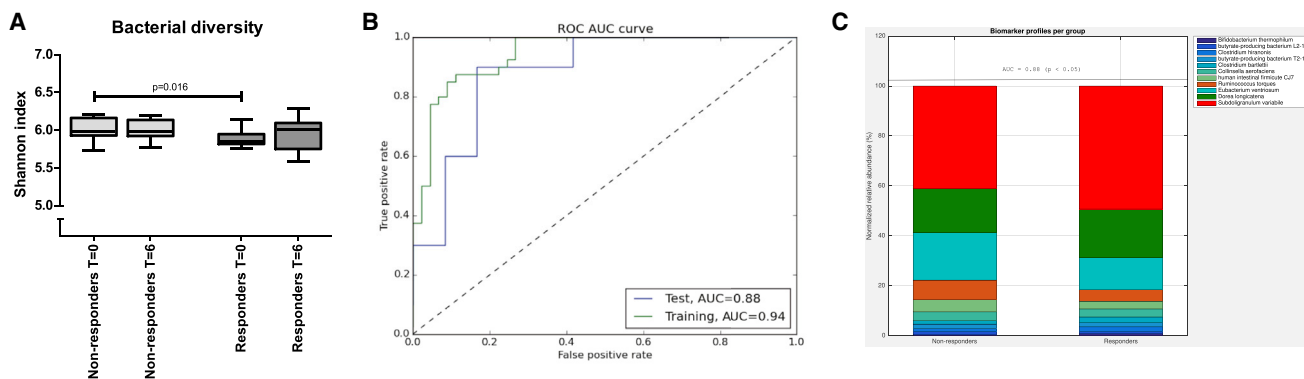


Figure 5. Fecal Bacterial Diversity and Bacterial Strains in Baseline Fecal Samples Related to Metabolic Response

(A) Shannon diversity index at weeks 0 and 6 for both Rd responders and non-responders is shown. Data are expressed as box-and-whisker plots. Significant differences ($p < 0.05$) are depicted.

(B) ROC of the model trained to predict response (Rd increase $\geq 10\%$ at 6 weeks after allogenic FMT) based on baseline fecal microbiota composition is depicted. (C) Bacterial species in baseline fecal samples that predict metabolic response are shown. The bar plots each depict a panel of 11 bacterial species in the baseline fecal sample of metabolic syndrome subjects that significantly can predict metabolic response upon allogenic FMT at 6 weeks. On the x axis metabolic response at 6 weeks is shown. On the y axis baseline relative abundance of each identified fecal bacterial species (L2 norm) is shown.

FMT, fecal microbiota transplantation; Rd, rate of (glucose) disappearance trained to predict responders (Rd increase $\geq 10\%$ at 6 weeks after allogenic FMT) or non-responders ($<10\%$ Rd increase) based on baseline fecal microbiota composition; AUC, area under the curve; ROC, receiver operating characteristic.

regulate engraftment of (lean-donor-derived) bacterial species and thus predict treatment success (Cotillard et al., 2013; Li et al., 2016). Disentangling such a specific signature of intestinal microbiota involved in beneficial functional (metabolic) shifts might help to apply approaches aiming to better predict development of insulin resistance and design targeted microbiota-based interventions in obese humans.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
 - Study Subjects
- **METHOD DETAILS**
 - Study Design
 - Sample Analyses
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
 - Multivariate Machine Learning Analysis

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and one data file and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2017.09.008>.

AUTHOR CONTRIBUTIONS

R.S.K., E.E.B., E.S.G.S., A.K.G., and M.N. designed the study. R.S.K., L.P.S., A.V.H., S.D.U., G.H., K.E.B., A.M.K., J.J.H., F.K.K., J.Z., H.S., A.C.H., T.H., J.J.G.H.M.B., H.A.R., F.G.S., S.W.M.O.D., M.T.A., G.M.D.-T., E.Z., and M.J.S. performed the research. R.S.K., E.L., and J.S. performed the statistical analysis. R.S.K., E.L., W.M.deV., E.S.G.S., A.K.G., and M.N. drafted the paper. All authors critically reviewed the manuscript.

CONFLICTS OF INTEREST

M.N. and W.M.deV. are founders and on the Scientific Advisory Board of Caelus Pharmaceuticals; E.L. is founder of Horaizon B.V.; and W.M.deV. is on the Scientific Advisory Board of Chr. Hansen, Hoersholm, Denmark, and the Nestle Institute for Health Science (NIHS), Lausanne, Switzerland. None of these possible conflicts of interest bear direct relation to the outcomes of this specific study.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PYY antibody	Statens Serum Institute Denmark	347-07
Total GLP-1	Statens Serum Institute Denmark	89390
Intact GIP	Statens Serum Institute Denmark	98171
Critical Commercial Assays		
HITCHIP	Agilent Technologies	N/A
High Pure PCR clean up kit	Roche	4983912001
	Stratagene	N/A
	Ambion	200339
RNAMAXX T7 Transcription Kit	Promega	N/A
	QIAGEN	AM8437
5-(3-Aminoallyl)-UTP	Merck	N/A
	GEhealthcare	M610A
	Sigma	N/A
RQ1 RNase-free DNase	Riedel-de Haen	N/A
RNeasy Mini-elute clean-up kit	Sigma	74204
	Sigma	N/A
	Agilent	N/A
Ethanol, > 99%	Agilent	N/A
	Invitrogen	N/A
	Invitrogen	RPN-5661
	Sigma	D8418
Post-labeling reactive Dye, Cy3 + Cy5	Sigma	N/A
	Sigma	N/A
	Sigma	31431
Dimethylsulfoxide, DMSO	N/A	S7277
	N/A	255580
	N/A	5185-5974
Na ₂ CO ₃ ·10H ₂ O	N/A	5188-5281
NaHCO ₃	N/A	15557-044
NH ₂ OH	N/A	24730-020
25x Fragmentation Buffer	N/A	93443-100ML
10x Blocking Agent	N/A	N/A
20x SSC	N/A	271004-1L
10% SDS	N/A	5758
10% Triton X-100	N/A	N/A
Acetonitril	N/A	N/A
Diethylpyrocarbonate, DEPC	N/A	N/A
Microarray scanner	Agilent	G2505C
SCFA analyses	Jasco Benelux (HPLC)	PU 4285
Bile acid analysis (plasma)	AB Sciex (LC/MS)	Api 3000
Bile acid analyses (feces)	Agilent GC MS	7890A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Bileacid analyses	AB Sciex	Analist 1.6
Statistical analyses	Prism	7
Microarray analyses	Agilent Feature Extraction software	Version 9.5
Other		
Dutch Trial Register	N/A	NTR 2705

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents (also see [Key Resources Table](#)) should be directed to and will be fulfilled by the Lead Contact, Max Nieuwdorp (m.nieuwdorp@amc.uva.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Study Subjects**

Male, omnivorous, Caucasian, obese subjects were recruited by newspaper advertisement and screened for characteristics of the metabolic syndrome. We included adult (age 21-69 years.) Caucasian males, who had obesity (body mass index (BMI) ≥ 30 kg/m²), fulfilled the National Cholesterol Education Program (NCEP)-criteria for metabolic syndrome ($\geq 3/5$: fasting plasma glucose ≥ 5.6 mmol/l, triglycerides ≥ 1.7 mmol/l, waist-circumference > 102 cm, high-density lipoprotein (HDL-)cholesterol < 1.03 mmol/l, blood pressure $\geq 130/85$ mmHg), were treatment naive and who were otherwise healthy. Exclusion criteria were a history of recent weight loss, cardiovascular event, cholecystectomy and the use of any medication known to influence gut microbial composition in the last three months (including proton pump inhibitors, antibiotics and pre-/pro-/synbiotics) or targeting metabolic diseases (e.g., lipid-lowering, anti-diabetic and/or anti-hypertensive drugs).

Lean (BMI < 25 kg/m²), omnivorous, healthy Caucasian males were also recruited by newspaper advertisements to serve as fecal donors. They completed questionnaires regarding dietary and bowel habits, travel history, comorbidity including (family history of) diabetes mellitus and medication use. Donors were screened for the presence of infectious diseases as previously published ([van Nood et al., 2013](#)). Blood was screened for presence of (antibodies to) human immunodeficiency virus; human T-lymphotropic virus; Hepatitis A, B, and C; cytomegalovirus (CMV); Epstein-Barr virus (EBV); strongyloides; amoebiasis and lues. Presence of infection resulted in exclusion, although previous, non-active infections with EBV and CMV were allowed. Donors were also excluded if screening of their feces revealed the presence of pathogenic parasites (e.g., blastocystis hominis, dientamoeba fragilis, giardia lamblia), bacteria (*Shigella*, *Campylobacter*, *Yersinia*, *Salmonella*, enteropathogenic *E. coli* and *Clostridium difficile*) or viruses (noro-, rota-, astro-, adeno (40/41/52)-, entero-, parecho- and sapovirus) at AMC department of Clinical Microbiology and Virology.

Written informed consent was obtained from all subjects. The study was approved by the local Institutional Review Board of the Academic Medical Center (AMC) in Amsterdam, the Netherlands, and conducted at the AMC in accordance with the Declaration of Helsinki. The study was registered at the Dutch Trial Register (number 2705).

METHOD DETAILS

We performed a double-blind randomized controlled trial (using computerized randomization) with male obese metabolic syndrome subjects. We studied the effect of allogenic (lean donor) gut microbiota infusion on metabolism in relation to intestinal microbiota composition 6 and 18 weeks after treatment, using autologous infusion as the control (placebo) treatment. Donors and recipients were randomly matched. In case a metabolic syndrome subject was randomized for 2 allogenic FMTs, the same selected lean donor provided the fecal sample for both FMTs.

Study Design

Participants were allowed to keep their own diet, but were asked to keep an online nutritional diary (<http://www.eetmeter.nl/>) to monitor daily caloric intake for seven days before each study week. During the 48 hours before the visits in weeks 0, 6 and 18, subjects collected 24h feces twice and stored this at home. In each study week, three successive study days were preceded by an overnight fast ([Figure S4](#)).

Study Day 1: Mixed Meal Test

The mixed meal test (MMT) started with insertion of an intravenous catheter in a distal arm vein, after which a baseline blood sample was drawn. After the baseline blood withdrawal, including fasting glucose, HbA1c, lipids and metabolites, subjects immediately ingested a standardized meal within 5 minutes. This meal contained 616 kCal (2.6 MJ), of which the energy content consisted out

of 66% fat, 33% carbohydrates and 6% proteins (Reijnders et al., 2016). Start of ingestion was considered $T = 0$ h and the following 4 hours, a blood sample was withdrawn for post-prandial metabolism (e.g., glucose, triglycerides, bile acids, enteroendocrine hormones) every 30 minutes. The blood samples were stored at -80°C .

Study Day 2: Two-Step Hyperinsulinemic Euglycemic Clamp Test and REE

Insulin sensitivity was measured during two-step hyperinsulinemic euglycemic clamp studies. We used $[6,6-^2\text{H}_2]\text{glucose}$ (> 99% enriched; Cambridge Isotopes, Andover, MA, USA) as tracer. After an overnight fast, catheters were inserted into a distal vein of both arms. One catheter was used for infusion of the glucose tracer, glucose and insulin, while the other was used for sampling of arterialized venous blood using a heated-hand box (60°C), respectively. At $T = -2$ h a blood sample was withdrawn for background enrichment, after which a continuous infusion of $[6,6-^2\text{H}_2]\text{glucose}$ (bolus $11\ \mu\text{mol kg}^{-1}$; continuous $0.11\ \mu\text{mol kg}^{-1}\text{ min}^{-1}$) was started and continued until the end of the experiment. After 2 hours of equilibration ($T = 0$ h) blood samples ($n = 3$, every 5 minutes) were drawn for isotope enrichments, glucoregulatory hormones and free fatty acid concentration. Directly thereafter, infusion of insulin (Actrapid; Novo Nordisk Farma, Alphen aan de Rijn, the Netherlands) was started at a rate of $20\ \text{mU m}^{-2}\text{ min}^{-1}$, meaning the start of step 1. Plasma glucose was measured every 10 minutes and 20% glucose enriched with 1% $[6,6-^2\text{H}_2]\text{glucose}$ (to approximate plasma enrichment) was infused at a variable rate to maintain plasma glucose at $5.0\ \text{mmol l}^{-1}$. After 2 hours of insulin infusion ($T = 2$ h), 5 repetitive blood samples were withdrawn every 5 minutes for glucose enrichments, gluco-regulatory hormones and free fatty acid concentrations. Hereafter, insulin infusion rate was increased to $60\ \text{mU m}^{-2}\text{ min}^{-1}$ for the second step. At $T = 4$ h another 5 blood samples were withdrawn as describe. Blood samples were stored at -80°C for later analyses. Resting energy expenditure (REE), carbohydrate- and fat oxidation were measured using indirect calorimetry during the basal and hyperinsulinemic state. Subjects also provided a fecal sample for gut microbiota and short chain fatty acid (SCFA) analysis on either day 1 or 2, and the sample was immediately frozen and stored at -80°C .

Study Day 3: Duodenal Biopsies with/without FMT

At the third study day, subjects reported to the gastroenterology ward in a fasting state for a gastroduodenoscopy followed by FMT according to a previously described procedure (Vrieze et al., 2012; van Nood et al., 2013). During the endoscopy procedure, we obtained duodenal biopsies, immediately collected in sterile tubes, snap-frozen in liquid nitrogen and afterward stored at -80°C , followed by positioning of a nasoduodenal tube. The position of the tube was assured by an abdominal X-ray immediately after the procedure. After ascertaining just placement of the tube, bowel lavage started with infusion of dissolved macrogol/electrolytes (Klean-Prep) via the tube at a rate of one liter/hour, until the gastro-intestinal tract was cleaned of fecal content. A maximum of 4 l of Klean-Prep was used. Prior to the duodenoscopy, every subject as well as the assigned lean donor provided fresh morning feces. At that moment subjects were randomized in a double-blind fashion to either allogenic (from lean male donors) or autologous (from own collected feces) FMT (Vrieze et al., 2012; van Nood et al., 2013). Based on the randomization procedure, the designated fecal sample was used within 6 hours after production for further processing by homogenizing the fecal sample with 500cc 0.9% sterile saline and filtering this solution through a metal sieve. The solution was stored in a sterile glass 500cc bottle. After the laxation procedure was completed, a total of 500ml of dissolved fecal content was inserted via the nasoduodenal tube.

All procedures were repeated after 6 weeks to study short-term effects of allogenic FMT. Thereafter, a randomized subgroup of patients received a second allogenic FMT (from the same lean donor) whereas the other subjects did not receive additional FMT. All subjects were studied for long-term effects of (either single or double) FMT at 18 weeks, repeating all aforementioned study procedures.

Sample Analyses

Fasting plasma lipids, including total cholesterol, low-density lipoprotein (LDL-) cholesterol, high-density lipoprotein (HDL-) cholesterol and triglycerides, were analyzed using commercially available assays (Diasys, Waterbury, Connecticut, USA) on a Selektro autoanalyser system (Sopachem, Ochten, the Netherlands). Plasma glucose and triglycerides were measured both pre- and during four hours post-prandially, in samples from the MMTs. Fasting plasma metabolites were determined by liquid chromatography-mass spectrometry (LC-MS) for a panel of 96 metabolites containing amino acids, oxidative stress markers and lipids as previously described (Hu et al., 2008; Noga et al., 2012; Fu et al., 2016).

Enteroendocrine Hormones

Plasma concentrations of the enteroendocrine hormones glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) were determined as previously described (Plamboeck et al., 2013), using pre- and post-prandial plasma samples from the MMTs. In short, plasma was treated with 70% ethanol (final concentrations) before analysis by RIA. PYY was measured using a mid-region specific antibody, code no. HYB 347-07 (Statens Serum Institut, Copenhagen, Denmark). Total GLP-1 was assayed using antiserum 89390, which has an absolute requirement for the intact amidated C terminus of the molecule. Intact GIP was measured using N-terminally directed antisera code nos 98171. Sensitivities were below $2\ \text{pmol/l}$ and intra-assay coefficients of variation better than 6% (Krupp and Holst, 1984; Plamboeck et al., 2005).

Bile Acids

Plasma bile acid concentrations as well as bile acid levels in 24h. feces were determined by mass spectrometry (respectively liquid chromatography-tandem mass spectrometry (LC-MS/MS)) or gas chromatography (GC)-MS. Plasma bile acid concentrations were determined in $25\ \mu\text{l}$ homogenized plasma, using pre- and post-prandial plasma samples from the MMTs. For sample preparation, $250\ \mu\text{l}$ internal standard solution containing D4-cholate, D4-chenodeoxycholate, D4-glycocholate, D4-taurocholate, D4-glycochenodeoxycholate and D4-taurochenodeoxycholate was added to the plasma. After mixing and centrifugation at $15.900\ \text{g}$ the supernatant

was transferred into a new vial. The supernatant was evaporated under nitrogen at 40°C. Bile acids were reconstituted in 100 μ l 50% methanol and filtered with a 0.2 μ m centrifugal filter at 3000 *g*. After this step, 10 μ l sample was injected into the LC-MS/MS system.

Fecal bile salt composition was analyzed in 24h. feces, for which subjects provided 24h. feces twice and we used the average results of both days. In short, 50 mg of dried feces was boiled in 1 ml of alkaline methanol (1M NaOH-methanol, 1:3 vol/vol) at 80°C for 2h after addition of 50nmol 5-cholestane and 14nmol 7,12-dihydroxy-5-cholanic acid as internal standard for neutral sterols and bile salts, respectively. After cooling down to room temperature, neutral sterols were extracted by using 3 \times 3 mL of petroleum ether, boiling range 60 – 80°C. The residual sample was diluted 1:9 with distilled water. A sample (100 μ l) of the solution was subjected to an enzymatic total bile salt measurement (Mashige et al., 1976). The remaining solution was used for bile salt isolation by reversed-phase solid-phase (C18) extraction (Mashige et al., 1976). The eluate was evaporated to dryness, and bile salts were derivatized to the methyl ester-trimethylsilyl derivatives for gas chromatography analysis. The extracted neutral sterols were derivatized to the trimethylsilyl derivatives by applying the same procedure that was used for bile salts. Bile salt composition of prepared fecal samples were determined by capillary gas chromatography on an Agilent gas chromatograph (HP 6890), equipped with a 25 m 0.25 mm CP-Sil-19-fused silica column (Varian, Middelburg, the Netherlands) and a flame ionization detector. The conditions were as follows: injector temperature 280°C; pressure 16.0 psi; column flow constant at 0.8 ml/min; oven temperature program: 240°C (4 min), 10°C/min to 280°C (27 min); detector temperature 300°C.

SCFAs

Fasting plasma levels of acetate, propionate and butyrate were determined in plasma samples, obtained at the MMT, using LC-MS, with minor modifications of the original method (van Eijk et al., 2009).

High-performance liquid chromatography (HPLC) analysis on fresh frozen stool samples for fecal SCFA determination was carried out according to the method from De Baere et al. (2013) with some modifications. Sets of 20 patient samples and 8 external calibration points including succinic acid as the internal standard (IS) were measured. All SCFA calibrator points and internal standard were prepared in milli-Q water. A 50 mM stock solution of the SCFA's containing acetic acid (AA), propionic acid (PA) and butyric acid (BA) was made, aliquoted and stored at –20°C until analysis. For the calibration curve double dilute series ranging from 25 – 0.39 mM were prepared and to each calibration point 50 μ l of IS (0.4 M) was added. The SCFA's from the patient samples were extracted from a 200–300 mg fecal sample. In brief, 1 mL Milli-Q Water was added, together with 50 μ l of IS, after which the samples were vortexed. 100 μ l of 12M HCl was added to all samples and calibration steps, after which they were vortexed for 15 s. Subsequently all samples were extracted twice with 7.5 mL di-ethylether for 20 minutes, centrifuged at 2000 *g* for 5 minutes and finally the supernatant was transferred into a clean 30 mL tube and 500 μ l of 1M NaOH was added. The aqueous phase (bottom) was then transferred to a new tube and 100 μ l of 37% HCl (12M) was added and vortexed. A 300 μ l sample was pipetted into a vial insert applicable for the HPLC autosampler. Of each sample 5 μ l was injected for HPLC analysis. The SCFA content was calculated by referring the SCFA/IS peak ratio to the standard curve. The SCFA concentration was expressed in μ mol/g feces. Separation of the SCFA was carried out on a Hypersil Gold aQ column (150 mm \times 4.6 mm, dp: 3 μ m) using a Jasco quaternary pump (PU4285, Jasco Benelux, De Meern, the Netherlands) at 30°C. Variable flow conditions were applied for the phosphate/acetonitrile gradient. UV detection was done at 210 nm using a Jasco UV detector (UV4075, Jasco Benelux, De Meern, the Netherlands). All samples were stored in a Jasco autosampler (AS4285, Jasco Benelux, De Meern, the Netherlands) at 4°C until analysis.

Insulin Sensitivity and REE

Plasma enrichment of [6,6-²H₂]glucose (tracer-to-tracee ratio), in samples obtained during the 2-step hyperinsulinemic euglycemic clamp tests, was determined by gas chromatography-mass spectrometry as previously described (Ackermans et al., 2001). Rate of appearance and rate of disappearance (Rd) of glucose were calculated using the modified version of the Steele equations for both steady-state (basal) and non-steady-state (during insulin infusion) measurements (Steele, 1959). EGP was calculated as the difference between the rate of appearance of glucose and the glucose infusion rate and expressed as μ mol kg^{–1} min^{–1}. Hepatic insulin sensitivity was determined by calculating the percentage suppression of basal EGP during the first step of hyperinsulinemia. Peripheral insulin sensitivity, expressed as Rd, was determined during the second step of hyperinsulinemia.

Resting energy expenditure (REE) was measured using indirect calorimetry. Oxygen (O₂) consumption (VO₂) and carbon dioxide (CO₂) production (VCO₂) were measured in the supine position during the basal and second step of hyperinsulinemia for 20 minutes using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA, USA). REE including carbohydrate and fatty acid oxidation were calculated as described previously (Frayn, 1983). The abbreviated Weir equation was used to calculate 24-hour energy expenditure (Weir, 1949).

Intestinal Microbiota

DNA from fecal samples was isolated using a combination of Repeated-Bead-Beating (RBB) and column purification, described in detail elsewhere (Salonen et al., 2010). Concentration and purity were assessed with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, North Carolina, USA). The microbial composition was determined using a previously described and benchmarked custom made, phylogenetic microarray, the Human Intestinal Tract Chip (HITChip; Data S1) (Rajilić-Stojanović et al., 2009). This is a custom-made Agilent microarray (Agilent Technologies, Palo Alto, CA, USA) containing approximately 5,500 specific oligonucleotide probes. In short, the full-length 16S rRNA gene was amplified, transcribed into RNA, labeled with the fluorescent dyes, Cy3 and Cy5 and hybridized to the array. Each sample was hybridized twice with a Pearson correlation of > 0.98 between replicates to ensure reproducibility and raw signal intensities were normalized as previously described (Lahti

et al., 2014). With some small adjustments, e.g., use of a more suitable reverse primer (Prok-1369-rev instead of Uni-1492-rev) and use of an accustart polymerase (Vrieze et al., 2012), a similar method was used to determine microbiota abundance in duodenal biopsies.

The HITChip microarray (Data S1) can detect 1,033 species-like bacterial phylotypes (level 3, > 98% 16S rRNA gene sequence similarity) that represent the majority of the microbial diversity in the human intestine. These were summarized to 130 genus-like phylogenetic groups (level 2, > 90% 16S rRNA gene sequence similarity) referred to as species and relatives ('*et rel.*'). The level 3 analysis may suffer from cross-hybridization issues, but has been used in several other studies to provide insight at the deepest possible level, as the analysis depth is comparable to approximately 200,000 16S rRNA reads with next generation sequencing (Claesson et al., 2009; Jalanka-Tuovinen et al., 2011).

Diversity of the microbiota was quantified based on non-logarithmized HITChip oligo-level signals by inverse Simpson's and Shannon's index using the Vegan package (Oksanen, Package 'vegan'. Community ecology package, version 2013). Probes were counted in each sample to measure richness by using an 80% quantile threshold for detection.

QUANTIFICATION AND STATISTICAL ANALYSIS

Our (hierarchical) hypothesis was that at 6 weeks after allogenic (lean donor) FMT, the peripheral insulin sensitivity (Rd) would improve by $5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (with a standard deviation of $4 \mu\text{mol kg}^{-1} \text{min}^{-1}$). Moreover, we also aimed to study whether a second lean donor FMT at 6 weeks, on top of the first treatment, would maintain this $5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ increase in Rd at 18 weeks. We expected that no extra treatment at 6 weeks (thus only single allogenic FMT at baseline) would result in Rd-levels at 18 weeks similar to baseline Rd-levels (exploratory analyses). With a randomization ratio of 2:1, the sample size should be 24 metabolic syndrome subjects treated with allogenic FMT, whereas we would need 12 metabolic syndrome subjects treated with autologous (own) FMT. Taking a dropout of 20% in each treatment arm into account, we aimed to include 45 metabolic syndrome subjects in total. With this sample size, the study has > 80% power in a 2-sided test with $\alpha = 0.05$.

Primary endpoint of the trial was the change in intestinal microbiota composition upon FMT in relation to insulin sensitivity. Other endpoints were changes in post-prandial lipid and glucose excursions, as well as plasma metabolites. A non-Gaussian distribution for all clinical data was assumed, and thus results are presented as medians and interquartile ranges. Post-prandial results (e.g., for plasma glucose, triglycerides, bile acids and enteroendocrine hormones) are described as (incremental) area under the curves (iAUC) for the 4 hour post-prandial follow-up, calculated by using the trapezoidal method.

Statistical testing was carried out using non-parametric tests. For between-group comparisons, either the Mann-Whitney U test or Kruskal-Wallis test was used. Friedman or Wilcoxon signed rank test was used for within-group comparisons of repeated-measurements. A false discovery rate corrected p value below 0.05 was considered significant, corrected for multiple testing in case of microbiota and metabolite data, as described underneath.

Multivariate Machine Learning Analysis

To study dynamics of biomarkers, e.g., species-level microbiota (level 3) and fasting metabolites, we computed the relative change for each individual subject over time. The relative change is, for example, the difference in microbial abundance between baseline and 6 weeks, divided by the microbial abundance at baseline, computed for each bacterial species per subject. In case of the microbiota analysis, this resulted into three datasets: 1) relative change in duodenal microbial composition of the allogenic and autologous treatment groups; 2) relative change in fecal microbial composition of the allogenic and autologous treatment groups; 3) relative change in fecal microbial composition of the responder and non-responder subjects. To assess the amount of change in intestinal microbial composition for each subject, we computed the magnitude of change by using L2 norm (Meyer, 2000). Informally, L2 (or Euclidian) norm is a measure of the vector length that is computed via calculating the sum of squared values of the relative differences of all species (between baseline and 6 weeks) per subject. The final result is calculated by taking the square root of the obtained value.

Biomarkers that allowed accurate discrimination among groups of subjects (allogenic versus autologous, responders versus non-responders) were selected by means of the elastic net algorithm (Zou and Hastie, 2005). Elastic net method is particularly applicable for the analysis of structured and high-dimensional data. It is a regularized method that combines the advantages of two techniques: LASSO (Tibshirani, 1996) (with variable selection property of reducing coefficients to zero values) and ridge regression (with shrinking coefficients to values for 'correlated trending' toward each other). This combination allows for the selection of the most important biomarkers, while taking the correlation (so called 'grouping effect') among them into account. Furthermore, by imposing an L1-penalty on the coefficients we obtained an interpretable model and viewed non-zero coefficients as the predictors that have the strongest predictive power. We used an adapted version of the elastic net algorithm (with Hinge loss function), which is specifically tailored for identification of the most important biomarkers (e.g., microbial species and metabolites) in the collected dataset, that jointly have an effect on differentiating between allogenic and autologous subjects as well as responders and non-responders. We trained the model by taking the gradient of the loss that is estimated at each sample at a time (stochastic gradient descent learning). Our statistic learning approach also includes stability selection (Meinshausen and Bühlman, 2010). While the biomarkers identified by elastic net algorithm usually lead to statistically significant results, they can frequently be unstable. In our approach, we address this problem via stability selection procedure (Meinshausen and Bühlman, 2010) coupled with the model selection. Biomarker stability is reflected in the frequency that a particular biomarker was identified in multiple simulations on a

re-randomized dataset. This procedure is especially relevant for small- to medium-sized data collections as recently published by our group (Botschuijver et al., 2017).

To avoid over-fitting, we used a 10-fold stratified cross-validation procedure over the training partition of the data (80%) while the remaining 20% was used as the testing dataset. Parameters to be selected are ratio between L1, L2 norms, and regularization threshold. Stability selection was performed by randomly subsampling 80% of the data 100 times. During stability selection procedure, all features having non-zero weight coefficient were counted. These counts were normalized and converted to stability coefficients having value between 1.0 for the feature that was always selected and 0.0 for feature which was never selected. We used Python (version 2.7.8, packages Numpy, Scipy) for implementing elastic net model and R (version 3.1.2) for visualization.

A randomization test was conducted to evaluate the statistical validity of the results obtained via elastic net algorithm. We followed the procedure where the outcome variable (e.g., allogenic versus autologous or responder versus non-responder) was randomly reshuffled while the corresponding microbial profiles were kept intact. This was repeated up to 100 times and Receiver-Operating-Characteristics Area-Under-Curve (ROC AUC) scores were computed each time. The performance measure used for a binary classification task is a ROC AUC. The ROC can be understood as a plot of the probability of correctly classifying allogenic versus autologous treated subjects or responders versus non-responders. Cross-validation within the dataset was accomplished by randomly hiding 20% of the subjects from the model and evaluating the prediction quality on that group. The ROC AUC score measures the predictive accuracy of the classification model with 0.5 AUC corresponding to a random result. A critical value of 0.05 was defined and the true AUC of the original dataset was compared with this value.

To visualize and study correlations among the subjects at different time points we used Redundancy analysis (RDA) (van den Wollenberg, 1977). RDA can be considered as a constrained version of principal components analysis (PCA), where the canonical axes are built from linear combinations of the response variables. RDA extends multiple linear regression by allowing regression of the response variables on multiple explanatory variables. Furthermore, to analyze the biomarkers of the subjects an unsupervised co-regularized spectral clustering algorithm was applied to the dataset (Tsivtsivadze et al., 2013; Biesbroek et al., 2014). In short, this multi-view clustering algorithm allows identification of clusters with similar microbial and metabolite profiles in an unbiased and robust manner. The method stems from a recently proposed class of multi-view clustering algorithms (Kumar et al., 2011) that have been reported to notably outperform standard techniques (e.g., k-means, hierarchical clustering, etc.) in accuracy and stability. Multi-view algorithms (closely related to cluster ensembles and consensus techniques) aim to combine multiple clustering hypotheses for increased accuracy and are not limited to a single similarity measure, thus leading to robust and reliable results.